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ORIGINAL ARTICLE

Rat Cerebellar Microanatomy and Neural Oxidative Redox Differentially Affected by Black Mustard Seeds Extract

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Abstract:

Background: Structural and functional alterations in cortical anatomy by environmental stressors are associated with several neuropsychiatric disorders. Many of these changes are supposed to fore start disease onset and result from environmental exposures. It was previously demonstrated that black mustard extract had a significant impact on the structural and functional integrity of the prefrontal cortex in our previous study. *Aim and Objectives:* To report effects of the aqueous extract of *Brassica nigra* (BN) black mustard seeds on the cerebellum of adult Wistar rats at varying doses. *Materials and Methods:* Twenty adult female rats weighing an average of 180 ± 20g were used. They were split into 4 groups (n = 5) and received administration orally; Group A (received extract at 200 mg/kg body weight), Group B (received extract at 100 mg/kg body weight), Group C (received extract at 50 mg/kg body weight), and Group D (received distilled water *ad libitum*). Rats from the experimental groups (A-D) were sacrificed 24 hours after the last day of administration which took 28 days, and cerebellar tissues were subjected to routine histochemical and biochemical analyses. *Results:* Spectrophotometric analyses of glutathione enzymes (GPx, GSH, GST) revealed that BN exerted an inverse dose-related comparative reduction in their neural activities in rats. Cytoarchitectural disposition of neural cells and evaluated Nissl bodies confirmed that BN at 100 mg/kg and 200 mg/kg dose initiated cell death within cerebellar sections of rat brains. Neurons in this brain region showed early signs of apoptosis, hallmarked by intercellular fragmentation, cytoplasmic shrinkage/ condensation and degradation of nuclear materials. *Conclusion:* Our results suggest that BN is not totally innocuous and thus, should be consumed in moderation.

Keywords: *Brassica nigra*, Cerebellum, Mustard Seeds, Neurotoxicity

Introduction:

Historically, plants have provided a source of inspiration for novel drug compounds, as plant-derived medicines have made a large contribution to human health and well-being [1]. Their role is twofold in the development of new drugs; They may become the base for the development of medicines; a natural blueprint for the development of new drugs and as phytomedicine to be used for the treatment of diseases [2]. Many of these plants are usually explored for their antioxidant properties, antioxidants which help in the scavenging of free radicals.

Mustard seeds are the small round seeds of a variety of mustard plants. The seeds are usually about one or two millimeters in diameter. They are important spices in many regional foods. The seeds can come from three different plants: black mustard [*Brassica nigra* (BN)], brown Indian mustard (*B. juncea*), and white mustard (*B.*

hirta/Sinapis alba) [3]. Together with, the seeds are widely acclaimed for many therapeutic conventions, including studies which indicate that N) seed has been used as a sedative for neurotic pain and rheumatoid arthritis, treatment of the brain and lung edema, paralysis, migraine and epilepsy [4, 5]. Experimental reports have also elucidated the antioxidant, hypoglycemic, anticancer and antimicrobial effect of BN seed [6, 7, 8, and 9].

The structural and functional integrity of the brain is largely dependent on an overlay of experiences, which might ultimately result in short-term or long-term/permanent pro-adaptive or maladaptive responses (altered susceptibility for psycho-pathology) [10]. It is of interest, therefore, to investigate the effects of a commonly used plant such as mustard seed (BN) on the cerebellum which is a region of the brain that plays an important role in motor control [11]. The cerebellum is said to be possibly involved in some cognitive functions such as attention and language, as well as in regulation of fear and pleasure responses [12]. The cerebellum does not instigate movement, but it ensures accurate timing, precision, and coordination [13]. Damages to the cerebellum, therefore, produces compromises in fine movement, equilibrium, posture and motor learning. Here we examined the impact of black mustard seeds (BN) extract on neural oxidative redox as well as cerebellar histomorphology.

Material and Methods:

Animal Care and Ethical Approval

All protocols and treatment procedures were carried out in strict compliance to the National Institutes of Health guide for the care and use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and according to Institutional Animal Care and Use Committee (IACUC) guidelines as approved by the College of Health Sciences Ethics Review Committee, University of Ilorin, Nigeria (19/08/2015).Wistar rats with an average weight of 185 ± 2g were procured from the Faculty of Veterinary Sciences, University of Ilorin, Nigeria and bred at the animal holding facility of the Faculty of Basic Medical Sciences, College of Health Sciences, University of Ilorin, Nigeria, where they had liberal access to rat chow and water.

Animal Grouping and Treatment

Twenty adult female rats were randomly assigned into 4 groups (A–D), each consisting of 5 rats (n = 5). The groups were treated as follows: Group A (received 200 mg/kg of extract daily for 28 days); Group B (received 100 mg/kg for 28 days); Group C (received 50 mg/kg for 28 days), and Group D (received distilled water for 28 days). The rats were weighed twice a week (72 h intervals), beginning from the first day of administration using a digital weighing balance. Percentage weight change for each rat was obtained by subtracting the initial weight (day 1 of administration) from final weight (24 h after last administration).

Purchase and Preparation of Extract

Mustard seeds were obtained from a local merchant in Ilorin. It was certified true by the Department of Plant Biology at the University of Ilorin. The extract was prepared according to the method described by Inyang *et al.* [14]. BN seeds were pulverized into powder form with an electric blender. Three hundred grams of the ground seeds were soaked in 1000 ml of distilled water; stirred and left for 72 hours in a refrigerator at 4°C. The mixture was sieved and filtered with Whatmann

No.1 filter paper. The resultant filtrate was dried in a water bath at 40°C for 96 hours to get the concentrate which was then diluted to stock.

Administration

The extract was administered via the oral route. Proper volume was ensured through the use of a calibrated syringe fitted with an oral cannula.

Sacrifice and Sample Collection

On completion of treatments, rats for histological analysis were euthanized using 10 mg/kg of ketamine (intramuscular route). Transcardial perfusion was done by exposing the left ventricle and injecting 50 ml of 0.1 M Phosphate Buffer Saline (PBS) (pH 7.4) followed by 400 ml of 4% Paraformaldehyde (PFA) while the rat was suspended in an inverted position (gravity). Excised brains were then rinsed in 0.25 M sucrose 3 times for 5 minutes each and then post-fixed in 4% PFA for 24 hours before being stored in 30% sucrose at 4°C until further processing. Rats for enzymatic assays were sacrificed by cervical dislocation, to avoid the interference of ketamine with biochemical redox; brains were then excised, rinsed in 0.25 M sucrose 3 times for 5 minutes each and placed in 30 % sucrose in which they were stored at 4°C. Coronal sections of cerebellum were obtained stereotaxically (+4 mm) from each brain. Histological staining was carried out in paraffin wax embedded sections which were stained with Haematoxylin and Eosin (H&E) using the methods described by Fischer *et al* [15]. Histochemical demonstration of Nissl substances was done with slight modification to the method published by Kádár *et al* [16].

Enzymatic Assays

Determination of the levels of Glutathione Peroxidase (GPx), Glutathione S-transferase (GST), Glutathione (GS), and Total Antioxidant Capacity (TAC) were carried out on cautiously dissected cerebellar cortices of treated rats using the spectrophotometric technique. Each of the assay kits was procured from Bio Legend Inc., San Diego, CA, USA. Equal weighing brain tissues (0.082g in sucrose at 4^{o}C) from rats across groups were pulverized in 0.25 M sucrose (Sigma) with the aid of an automated homogenizer at 4°C. Lysates from the brain were centrifuged for 10 minutes in a microfuge at 12,000 rpm to obtain the supernatant containing organelle fragments and synaptosomes. The supernatants were aspirated into plainlylabeled glass cuvette placed in ice. GPx, GST, GS, and TAC activities were assayed according to the manufacturer's instruction in the assay kit pack.

Light Microscopy

Cerebellar sections on glass slides were captured using an Olympus binocular research microscope (Olympus, New Jersey, USA) which was connected to a 5.0MP Amscope Camera (AmscopeInc, USA).

Data Analysis

All quantitative data were analyzed using GraphPad Prism® (version 6) and SPSS (version 20) software. Body weight, brain weight, GPx, GST, GS, and TAC outcomes were plotted in ANOVA followed with Tukey's multiple comparisons test. Significance was set at $p<0.05^{*}$ and $p<0.01^{\#}$. The results were represented in bar charts with error bars to show the Mean and Standard Error of the Mean (Mean±SEM) respectively.

Results:

Body and Brain Weight Changes

As already aforementioned in the methods, the body weight of the animals was taken with a digital weighing scale on weekly basis to monitor the changes observable for the period of administration. At the end of the first week of administration, there was a statistically significant increase in the weight gained by animals in the 200 mg/kg body weight and 100 mg/kg body weight dosage groups, $p<0.05$, when compared with the control (Table 1). There was also found to be an increase in weight gained by the low dose group of 50mg/kg body weight group in comparison with the control group. However, this increase was not enough to be statistically significant. The following weeks of administration saw subtle increases in the weight of each experimental group (Table 1).

Indirect Proportionality of BN dose versus Neural Antioxidant Status

The effect of the three varied doses on neural antioxidant levels was measured GPx is a selenium-containing antioxidant enzyme that effectively reduces Hydrogen Peroxide (H_2O_2) and lipid peroxides to water and lipid alcohols, respectively, and in turn, oxidizes glutathione to glutathione disulfide. The level of GPx showed an indirect relationship with the dose of the extract as it was observed that the levels of glutathione decreased as the dose increased (Fig. 1). The increase in the level of glutathione peroxidase was significant in the low dose (50 mg/kg) group when compared with the 200mg/kg dosage group (Fig.1). Similar were the observations in the comparative level of Reduced Glutathione (GSH) across treatment groups, which also showed an indirect relationship with the dose of the extract as

Table 1: Showing the Pattern of Weight Change in All Animal Groups. The First Week of Administration saw a Statistically Significant Increase in the Body Weight of Animals in Group A (200 mg/kg b w of BN Extract) and Group B (100 mg/kg b w of BN Extract) Relative to the Control. Subsequent Changes in Weight of Experimental Animals were Statistically Insignificant Comparatively.

Groups	Week 1	Week 2	Week 3	Week 4
A. BN extract 200 mg/kg bw	203.00 ± 38.50*	218.00 ± 50.81	220.00 ± 53.26	224.60 ± 50.08
B: BN extract 100 mg/kg bw	199.00 ± 22.19*	205.00 ± 23.97	210.00 ± 28.50	214.00 ± 28.84
C: BN extract 50 mg/kg bw	195.00 ± 20.91*	196.00 ± 21.03	206.00 ± 23.82	209.40 ± 23.86
D: Control	176.00 ± 31.89	189.00 ± 25.34	205.00 ± 23.18	211.00 ± 23.02

*Values are Represented as Mean ± SEM, * is the Level of Significance at $p<0.05$.*

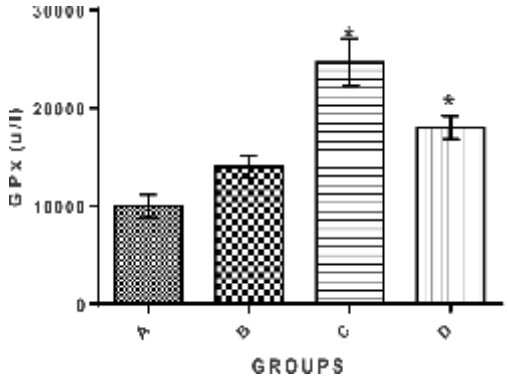

Fig. 1: Chart Showing Changes in the Levels of GPx

*A= BN Extract 200mg/kg b w, B= BN Extract 100mg/kg b w, C= BN Extract 50mg/kg b w, D= Control Group.BN treatment at 200mg/kg revealed marked depletion in brain GPx levels ($p<0.05$) relative to the control. * is the level of significance at $p<0.05$in comparison to group D.*

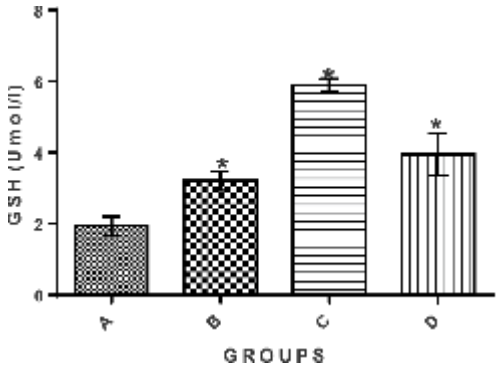

Fig. 2: Chart Showing Changes in the Levels of GS

*A= BN Extract 200 mg/kg b w, B= BN Extract 100 mg/kg b w, C= BN Extract 50 mg/kg b w, D= Control Group. BN Treatment at 200mg/kg Revealed Marked Depletion in Brain GSH Levels ($p<0.05$) Relative to the Control. * is the Level of Significance at $p<0.05$in Comparison to Group D.*

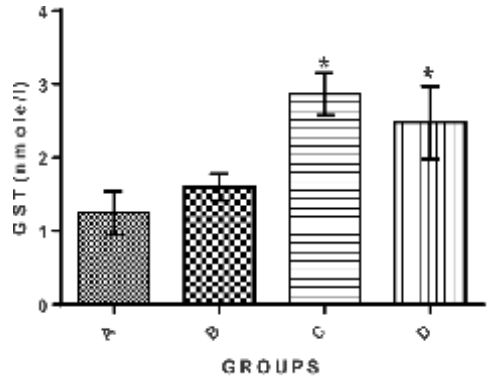

Fig. 3: Chart Showing Changes in the Levels of GST

*A= BN Extract 200 mg/kg b w, B= BN Extract 100 mg/kg b w, C= BN Extract 50 mg/kg b w, D=Control Group. BN Treatment at 200 mg/kg Revealed Marked Depletion in Brain GST Levels ($p<0.05$) Relative to the Control. * is the Level of Significance at $p<0.05$ in Comparison to Group D.*

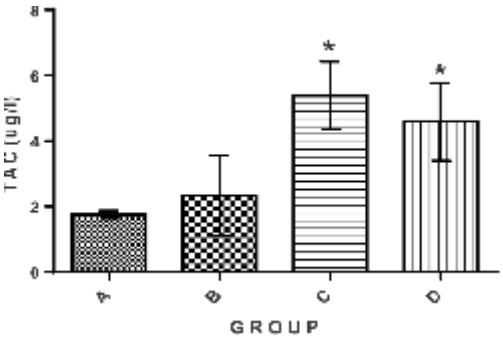

Fig. 4: Measurement of TAC of BN in Cerebellar Lysates

*A= BN Extract 200 mg/kg b w, B= BN Extract 100 mg/kg b w, C= BN Extract 50 mg/kg b w, D=Control Group. * is the Level of Significance at $p<0.05$ in Comparison to Group D.*

it was observed that the levels of glutathione decreased as the dose increased (Fig. 2). This result further corroborated what we observed with GPx levels.

GSTs are enzymes that catalyze the reaction of GS with electrophiles of both endogenous and xenobiotic origins. To verify our results, we examined the turnovers of GST across experimental groups. Likewise, the increment in the presence of BN (dose-wise), showed statistically significant ($p < 0.05$)dose-related decreases in an inverse manner (Fig. 3). So far, our results have pointed out one salient point vis; glutathione activity status following BN administration seem to undergo depletion past a particular dose threshold. Visual increments are seen in the low dose (50 mg/kg) group when compared with the control group.

TAC of BN in the Cerebellum

We further decided to comparatively measure the TAC in each experimental group. Figure 4 shows that the TAC was lowest in the group that received the highest dose of the extract followed by the group that received 100 mg/kg body weight. The reduction in the levels of total antioxidant capacity was significant ($p < 0.05$) in these two groups when compared to the control group. There was a subtle increase in the total antioxidant capacity in the low dose group (50 mg/kg) which was not significant when compared to the control group.

BN-induced Cerebellar Histomorphological Alteration

Demonstrating cytological and structural disposition of cells reveals specific show cases resulting from physicochemical alterations induced by BN administration. In this experiment, cerebellar morphology was demonstrated using histological (H&E staining) and histochemical (Cresyl violet staining) techniques. Animals treated with distilled water in Fig. 5d and 6d did not show any pathologic alteration in the morphological appearance of cerebellar cells and the neuropil which were aptly stained. The layering of cells from the Molecular Layer (ML) to the Granular Layer (GL) is generally normal and structurally well-defined. Similarly, on observing the Purkinje (P) cells using higher power magnification, distilled water, and 50 mg/kg treatment did not alter the structural integrity of neurons (Fig. 5c and d). Also, apical and basal dendritic spines were well expressed and interconnected within the neuropil of both groups. However, 100 mg/kg and 200 mg/kg treatment groups showed several degenerative changes within cerebellar sections (Fig. 5a and b). In these groups, cerebellar histomorphology was characterized by fragmentation of neuropil, distortion of layering, infiltration of granule cells into the Purkinje layer and vice versa, and cytoplasmic degeneration of neurons. Corroboratively, there was a marked reduction in the deposition of Nissl proteins in cerebellar sections of these groups when compared to rats that received distilled water and BN at 50 mg/kg dose, which is indicative of degenerative changes via alteration in protein synthesis (Fig. 6).

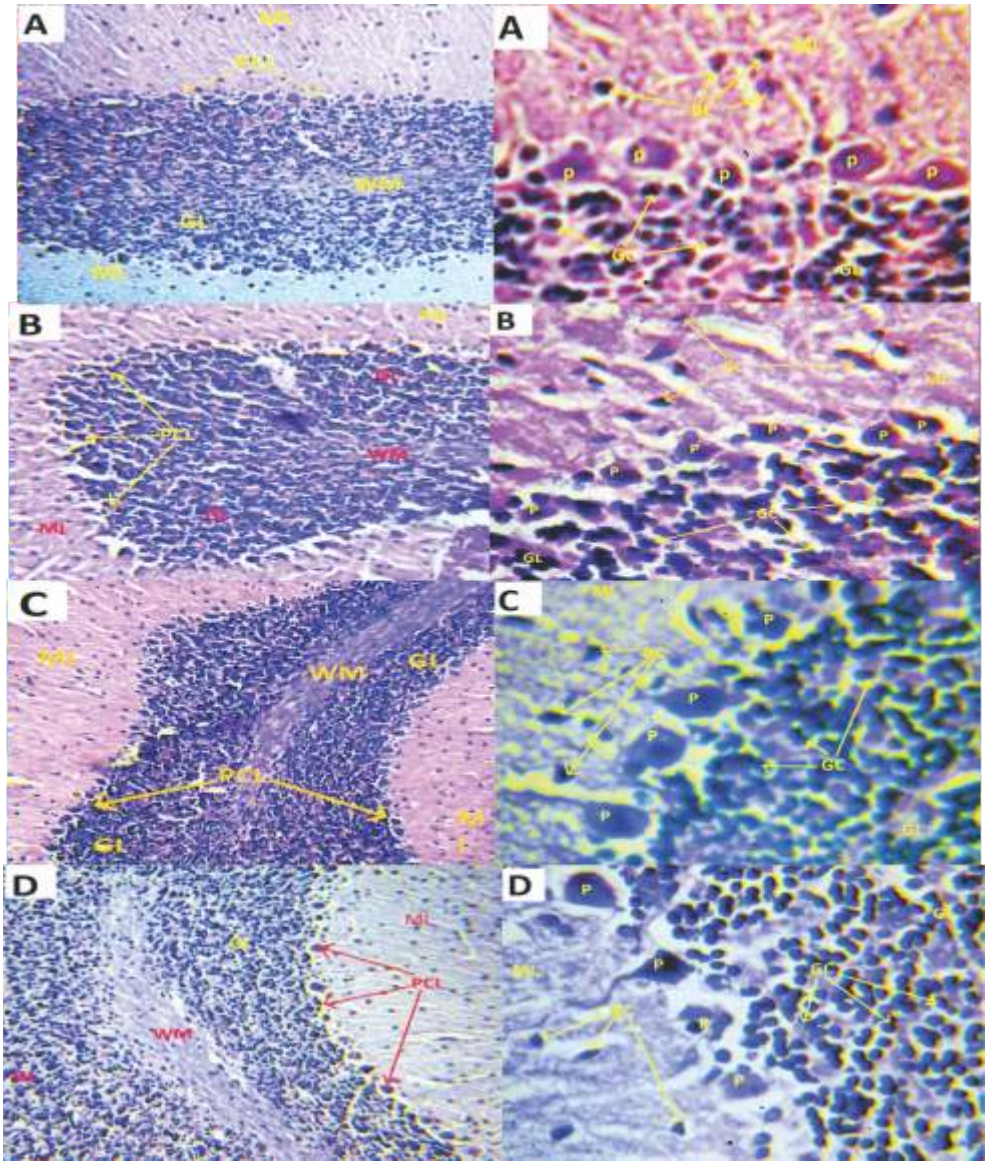

Fig. 5: Histological Analyses of Cerebellar Morphology of Rats. **ML**= Molecular Layer, **GL**=Granular Layer, **PCL**= Purkinje Cell Layer, **GC**=Granule Cells, **BC**= Basket Cells, **WM**= White Mater. **A**= 200 mg/kg, **B**=100mg/kg, **C**=50mg/kg and **D**=control.
H&E Stain at x40 and x400 Magnification shows Panoramic View and High Power Magnification of the General Histology Cerebellum of Treated Rats. Deeply Stained and Characteristically Normal Cellular Layering of the Granule Cell Layers with Well-outlined Purkinje Cell Layers can be seen in Control and 50mg/kg Dose Groups. Purkinje Cell Layer in the 100 mg/kg and 200 mg/kg dose Groups Appears Distorted and Poorly Outlined in the Lower Magnification. At A Higher Magnification (×400),Purkinje Cells (Red Arrows) with Axons Jetting into the Molecular Layer is Well Delineated in Control and 50 mg/kg Groups

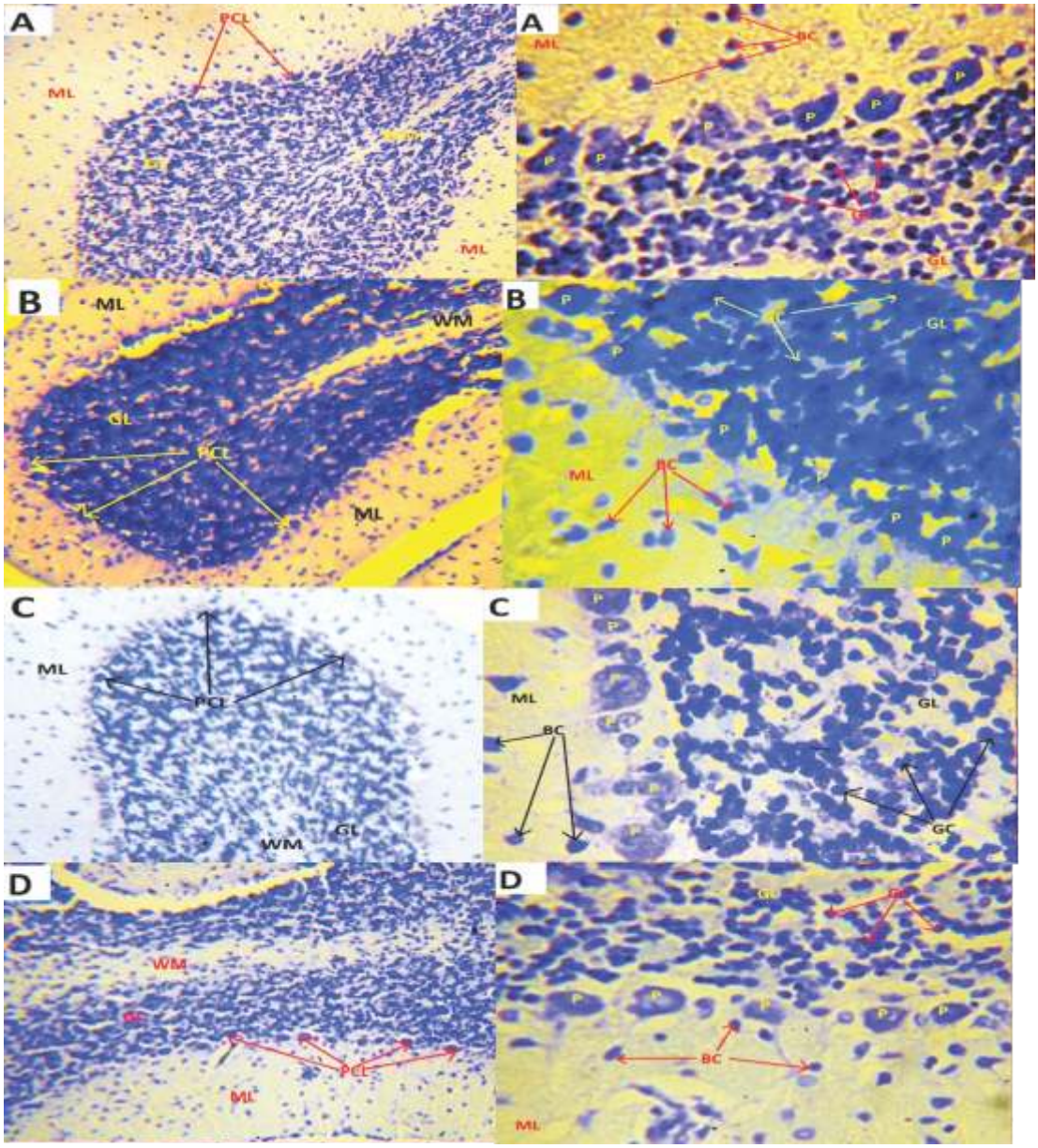

Fig. 6: Histochemical Demonstration of Cerebellar Morphology in Treated Rats shown in Cresyl Fast Violet for Nissl Substance. ML= Molecular Layer, **GL**=Granular Layer, **PCL**= Purkinje Cell Layer, **GC**=Granule Cells, **BC**= Basket Cells, **WM**= White Mater. **A**= 200 mg/kg, **B**=100mg/kg, **C**=50mg/kg and **D**=control.

Cresyl Fast Violet at x40 and x400 magnification. At a Panoramic View (×100), All the Micrographs Appear to have The Same Staining Intensity and Cellular Density within the Granular Layer. Meanwhile, at a Higher Power Magnification (×400), 200 mg/kg Group Present with Chromatolytic Purkinje Cells

Discussion:

We have extended our previous study of the dose-dependent effects of BN (black mustard seeds) extract on neural spatial regions of Wistar rats [17]. Here, our focal point was cerebellar structural integrity and oxidative redox. The results demonstrated a progressive decrease in the expression of different key antioxidant enzymes with increasing BN dose in the brain of rats. Indeed, reduced glutathione plays a major role in the regulation of the intracellular redox state of vascular cells by providing reducing equivalents for many biochemical pathways [18]. In the absence of adequate GPx activity or glutathione levels, H_2O_2 and lipid peroxides are not detoxified and may be converted to Hydroxyl(OH^-) radicals and lipid peroxyl radicals, respectively, by transition metals (Fe^{2+}). It should be noted that glutathione is essential for the activity of GPx since GPx converts reduced glutathione into oxidized GS [19]. This might explain the coordinated decrease in cellular glutathione content and GPx expression induced by BN extract at 100 mg/kg and 200 mg/kg. The slight increments witnessed in the 50 mg/kg treated group when compared with the control, may be an adaptive response to oxidative stress [18, 19]. The main biological roles of GSTs encompass detoxification and protection against oxidative stress. By conjugating glutathione with toxic electrophilic substrates, the resulting molecules generally become less reactive and more soluble, thus facilitating their excretion from cells and the organism [20]. Spectrophotometric analyses of glutathione enzymes revealed that BN exerted a dose-related comparative reduction in their neural activities in rats. These observations, however, are in contrast with Badrul *et al.* [21], where the antioxidative properties of BN were shown to increase. Kiasalari *et al.* [8] also noted that SOD level increased in animals treated with 150mg/kg body weight of hydro-alcoholic BN seeds extract in their experiment on evaluating the antiepileptic and antioxidant effect of BN on pentylenetetrazol-induced kindling mice [8].

The cytoarchitectural disposition of neural cells and evaluated Nissl bodies confirmed that BN at 100 mg/kg and 200 mg/kg dose initiated cell death within cerebellar sections of rat brains. Neurons in this brain region showed early signs of apoptosis, hallmarked by intercellular fragmentation, cytoplasmic shrinkage/condensation and degradation of nuclear materials. These changes have been attributed to oxidative impairment to DNA, which can be induced toxic materials, particularly by increasing the free radical incidence and thereby leading to the generation of oxidative stress. Results revealed by the histological sections showed a variation in cerebella layering across the experimental groups. The Purkinje cell layer was avidly separating the molecular layer from the granular layer in the group C (50mg/ kg of mustard seed extract) as also seen in the control group. The distinctiveness of the Purkinje cell layer reduced as the dose of the extract increased. This distortive expression is most probably caused by the induction of oxidative stress as the dose of mustard seed extract increased, which will lead to an increase in the levels of Reactive Oxygen Species (ROS). ROS such as superoxide anion (O^{2-}), singlet oxygen (O^-), OH^- and H_2O_2 generated by actions of excess mustard seed extract probably disrupted the balance between defensive and aggressive factors

maintaining the integrity of cellular layers [22]. These high levels of ROS scavenge electrons from the lipid bilayer of the cell membrane thereby causing a destabilized cellular morphology and cytoarchitecture [23, 24, and 25]. Free radicals or ROS are highly reactive, unstable molecules that oxidize cellular components such as DNA, proteins, lipids/fatty acids and advanced glycation end products (e.g. carbonyls) resulting in damage to the cell membrane, mitochondrial apparatus and the DNA [26, 27].

The staining intensity for Nissl substance also showed a variation across the experimental groups. The control group and group C (50mg/kg of mustard seed extract) animals showed an intensive affinity for the stain, evident by the Nissl substance of cells of the Purkinje cell layers showing apparent cytoplasmic processes projecting into the molecular layer. The Nissl substances of the basket cells in the molecular layer and those of the granule cells of the molecular where also intensively stained. This observation is indicative of unaltered synthesis of proteins in the protoplasm of cells. The cytoplasmic processes of the Purkinje cells became less conspicuous as the dose increased (100 mg/kg and 200 mg/kg doses). It is important to note that the end-point of afferent pathways to the cerebellar cortex is the characteristic Purkinje cell [11]. The modulation of cerebellar output also occurs at the level of the Purkinje cells which may be responsible for the motor learning aspect of cerebellar functions [11, 28]. Therefore, an affectation of Purkinje neurons through ingestion of BN at100 mg/kg and 200 mg/kg may resort into circuit degeneration within the cerebellum of rats, which will furthermore result in altered motor activities. The granule cells also became aggregated together with infiltration of the cells into outer layers as the dose increased, perhaps as a result of distortion of the cytoplasm as a result of the stress-inducing actions of the increasing doses.

Conclusion:

In conclusion, our study showed that BN possesses potent phytoactive constituents that can induce morphological and functional alterations in the cerebellum. A major highlight of the potentials of BN is seen in its ability to down-regulate the antioxidant status of neuronal cells at 100 mg/kg and 200 mg/kg doses.

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